

High IFN- γ Production of Individual CD8 T Lymphocytes Is Controlled by CD152 (CTLA-4)¹

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CD8 T cell expansion and cytokine production is needed to generate an effective defense against viral invasion of the host. These features of CD8 T lymphocytes are regulated, especially during primary responses, by positive and negative costimulation. We show in this study that surface expression of CD152 is highly up-regulated on activated CD8 T lymphocytes during primary immune responses, suggesting a prominent regulatory role. Indeed, production of the proinflammatory cytokine IFN- γ , but not TNF- α , by CD8 T cells was inhibited by CD152 engagement. The inhibition was regulated independent of proliferation and IL-2 production, but dependent on the quality of the TCR signaling. We show that signals induced by CD152 on activated CD8 T lymphocytes reduce the frequency of IFN- γ^{high} -expressing cells. Our data also show that in activated CD8 T cells, the CD152-mediated inhibition of cytokine production is more pronounced than inhibition of their proliferation. *The Journal of Immunology*, 2007, 178: 2132–2140.

CD8⁺ T lymphocytes play a crucial role in protection from intracellular pathogens such as viruses. The key effector cytokine produced by CD8 T cells is IFN- γ . Pleiotropic actions of IFN- γ induce diverse effects on a wide range of target cells that include induction of both specific and nonspecific mechanisms of host defense against infectious agents and tumors. IFN- γ is one of the major cytokines responsible for up-regulating MHC class I and II proteins on a variety of cells such as macrophages and epithelial cells (1, 2). In addition, this cytokine regulates humoral immune responses by affecting IgG H chain switching in both a direct and indirect manner (3, 4). It has been shown that IFN- γ produced by CD8⁺ T cells acts directly on CD4⁺ Th1 differentiation (5). In addition, IFN- γ regulates the production of a variety of other proinflammatory cytokines including IL-12 and TNF- α and stabilizes inflammatory T cell responses (5, 6). Disruption of IFN receptor γ -chain expression in mice in vitro and in vivo impaired the ability of these mice to resist infections by a variety of microbial pathogens including *Listeria monocytogenes*, *Leishmania major* and several species of mycobacteria despite the fact that the mice developed normal helper and cytotoxic T cell responses to these pathogens (7–9). This result demonstrates that IFN- γ plays a critical role in the expression of innate host resistance to microbial infections. These studies also clearly identified an important role for IFN- γ in promoting tumor immunogenicity

(10). Generally, proinflammatory cytokines are a major control switch to initiate as well as maintain inflammatory responses.

CTLA-4 (CD152) is a major down-regulator of immune responses (11, 12). Although CD152 was discovered initially as mRNA that is preferentially transcribed in CD8⁺ T cells, it can also be detected in activated CD4⁺ lymphocytes (13, 14). The inhibitory role of CD152 became strikingly evident in CD152-deficient mice, which suffer from a lymphoproliferative disease of highly activated CD4 T lymphocytes. Under suboptimal stimulatory conditions, CD152 probably inhibits the early stages of TCR signaling in general by preventing formation of membrane lipid rafts, inhibiting accumulation of IL-2 mRNA, preventing nuclear translocation of the transcription factor NF-AT, inducing G₁ cell cycle arrest in an IL-2-independent manner, and diminishing expression of activation-associated receptors such as CD69 and CD25 (11, 15, 16). In addition, at the peak of surface CD152 expression on activated CD4 T cells, CD152 regulates expansion of these proliferating cells and induces resistance against apoptosis (17, 18). CD8 T lymphocytes are not affected in CD152^{-/-} mice, which implies a different effect of CD152 on CD4 and on CD8 T cells. Moreover, it has been shown that CD152 is involved in inducing anergy in CD4 T cells (19), but not in CD8 T cells, rather CD152 has been shown to function synergistically with programmed death-1 (20, 21). Thus far, detection of CD152 on the surface of primary T cells by conventional techniques has been difficult due to low levels of surface expression. In MHC class-I-restricted CD152^{-/-} animals, like in 2C-TCR^{tg} mice, CD8 T cells maintain a naive phenotype in young animals (22). Nevertheless, CD152 is expressed and is functional at the cell surface on activated CD8 T cells from wild-type (WT)⁵ and 2C-TCR^{tg} mice (14, 23). Proliferation in response to anti-CD3 and anti-CD28 in vitro can be inhibited by CD152 cross-linking (11, 14, 24). Previous studies imply that CD152 regulates secondary recall responses but not primary immune responses (22), suggesting that CD152 plays

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⁵ Abbreviations used in this paper: WT, wild type; KO, knockout; MFI, mean fluorescence intensity; neg, negative.

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a regulatory role only in differentiated CD8 lymphocytes. Specifically, expansion but not differentiation is regulated by CD152, which leads to an overall reduction in cytokine secretion due to reduced numbers of effector cells, but not due to reduced cytokine secretion of individual cells (22). In addition, it has been shown that CD152 signaling on CD8 T lymphocytes has no significant function *in vivo* (25, 26). Therefore, regarding primary CD8 T lymphocyte responses, the precise function of CD152 is largely undefined.

In this study, we investigated the functional role of CD152 in primary CD8 T cells upon antigenic or polyclonal stimulation. We show here that CD152 differentially regulates IFN- γ production but not cell cycle progression and that inhibition of the proinflammatory cytokine IFN- γ is regulated by CD152 engagement in a cell autonomous manner. This novel function of CD152 activation on CD8 T cells may be important in preventing potentially hazardous inflammatory reactions while maintaining viral and tumor immunogenicity.

Materials and Methods

Mice

OVA-specific TCR^{tg} mice (OT-1 and OT-2) both CD152^{WT} and CD152^{-/-}, lymphocytic choriomeningitis virus-specific TCR^{tg} mice (P14), and C57BL/6 mice were bred under specific pathogen-free conditions in the animal facility of the Bundesgesundheitsamt (Berlin, Germany) or in Institute of Experimental Immunology (Zürich, Switzerland). All mice were used at the age of 5–8 wk, and offsprings of OT-1, CD152^{-/-} mice were genotyped by PCR using oligonucleotide primers (CD152, 5'-TGGAGTCCTTCATAGTTAGG and CD152, 3'-GCAAGATGGTGAGTGTGATGTT; Neo, 5'-CATAGTTAGGTCTGTGATGC and Neo, 3'-CGTCAAGAAGGCGATAGAAGGC). All mice have been backcrossed onto the C57BL/6 strain for >10 generations. All animal experiments were performed with age- and sex-matched controls in accordance with institutional, state, and federal guidelines.

Abs, cytokines, and reagents

The following Abs against murine Ags were used: anti-CD152 (UC10-4F10-11), V α 2-TCR (B20.1), and IFN- γ R (2E2) (BD Biosciences) in their respective form of FITC, PE-, Cy5-, or biotin-conjugates. Annexin V-PE was purchased from BD Biosciences. Anti-CD3 (145-2C11), anti-CD8 (196), anti-CD44 (IM7), anti-CD62L (MEL14), and anti-IFN- γ (AN18.17.24) were purified from hybridoma supernatants with protein G and controlled by HPLC, PAGE, and FACS analysis. Control Abs such as IgG1 (R3-34) and FITC-conjugated hamIgG2 (anti-KLH, HA4/8) were purchased from BD Biosciences. Magnetic microbeads anti-CD8, anti-CD62L, anti-CD90, and anti-FITC were purchased from Miltenyi Biotec. Murine IL-2 was used from culture supernatants of transfected mouse cell line X63-IL-2.6. Concentration and bioactivity was determined by ELISA and IL-2-dependent mouse cell line CTLL-2. Abs and Fab were controlled using HPLC analysis showing routinely only one peak. Neutralizing anti-CD152 Fab were prepared from whole Abs with Immunopure Fab preparation Kit (Pierce) and were used at 200 μ g/ml in cell cultures. Specificity of anti-CD152 Abs were controlled by FACS staining. Toxicity and function of Fab was tested in Ag-stimulated T cell cultures using trypan blue. Anti-CD152 Fab were used when at least twice as much CD4 T lymphocytes were detected upon Ag-specific stimulation in cultures treated with anti-CD152 Fab compared with cultures treated with control Fab. PMA, ionomycin, and brefeldin A were purchased from Sigma-Aldrich and used in following concentrations: PMA (10 ng/ml), ionomycin (0.5 μ g/ml), and brefeldin A (10 μ g/ml).

Preparation of liposomes

Large unilamellar vesicles were prepared as described by Hope et al. (27). Briefly, 300 μ mol of a mixture of lipids (molar ratio DPPC:Chol:DPPG:PDP-DPPE = 45:40:10:5) dissolved in chloroform and glass beads were deposited in a glass vessel. Additionally, we added the fluorescent lipophil dye Cy5 (D7757; Molecular Probes) in a concentration of 0.23 μ M. The solvent was allowed to evaporate and the remaining lipid film was dried under vacuum for 1 h at 65°C. The lipids were then hydrated at 65°C with ~4 ml of PBS (pH 7.5) containing 10 mM chloroform and magnetic beads. The suspension was submitted to several freeze-thaw cycles and extruded 5–10 times through filters of 800- and, subsequently, 400-nm pore size

(Millipore). Liposomes were separated from free magnetic particles by centrifugation over a Ficoll (Pharmacia) density gradient. Liposomes containing magnetic particles were then isolated by high-gradient magnetic field filtration, with the MACS system (Miltenyi Biotec). Size distribution was estimated by measuring the relative fluorescence intensity of the different liposome fractions by flow cytometry.

Conjugation of Abs to liposomes

Anti-digoxigenin Fab (1 mg/ml in carbonate buffer; pH 8.5) were modified with DTT. The product was isolated by gel chromatography on a Sephadex G20 column (PD10; Pharmacia), equilibrated with PBS. The modified protein was stored at -20°C until used. For conjugation, the liposomes were first activated with NHS-sulfo-SMCCm and then incubated with the modified protein at a final concentration of 0.5 mg/ml and gently stirred for 1 h. Unconjugated protein was then removed from liposomes by gel chromatography on Sepharose CL4B (Pharmacia).

Cell isolation and stimulation

Isolation of naive CD8⁺CD44^{low} T cells was performed using two-parameter high-gradient MACS and FACS. Splenocytes from OVA-specific TCR^{tg} mice were incubated with anti-CD8 magnetic microbeads according to the manufacturer's instructions and enriched to a purity of >95%. Subsequently, they were sorted by FACS DiVa (BD Biosciences) for CD44^{low} phenotype with mAb anti-CD44-conjugated to FITC or Cy5. By this procedure, CD8⁺CD44^{low} cells were purified to >99.9% as determined by flow cytometry. Isolated cells were stimulated either with 1 μ g/ml SIINFEKL (OVA₂₅₇₋₂₆₄) peptide (Mimotopes) or with anti-CD3 (2 μ g/ml) and APCs at a final cell density of 3 \times 10⁶ cells/ml, the medium being RPMI 1640 with added 10% FCS, 0.3 mg/ml glutamine, and 10 μ M 2-ME. T cell-depleted splenocytes from congenic C57BL/6 mice were used as APCs such that CD8:APC ratio was 1:6. Cells were allowed to proliferate and were expanded with IL-2 (50 U/ml) added during splitting of cells on day 3 after primary stimulation. Secondary stimulation was done on day 9–12 after primary stimulation with 1 μ g/ml SIINFEKL (OVA₂₅₇₋₂₆₄) peptide, APCs, and IL-2 (50 U/ml). Isolation of naive CD62L^{high}CD4⁺ T cells was performed using MACS. Splenocytes from OVA-specific TCR^{tg} mice were incubated with FITC-conjugated anti-CD4 mAb and subsequently with MultiSort anti-FITC microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD4⁺ cells were allowed by positive selection on an autoMACS system (Miltenyi Biotec) to a purity of >99.3%. After the release of MultiSort anti-FITC microbeads, CD4⁺ cells were incubated with anti-CD62L microbeads. CD62L^{high}CD4⁺ cells were positively selected on autoMACS to a purity of >99% as determined by flow cytometry. Isolated cells were stimulated with 1 μ g/ml OVA₃₂₃₋₃₃₉ peptide (Neosystem) and APCs and expanded as described for CD8 T cells above. Cell stimulation was done with indicated concentrations of peptide or Ab as described above unless otherwise specified. T cells were removed by depletion of CD90⁺ cells after incubation with anti-CD90 microbeads and subsequent negative selection. To ensure that an equal number of CD8 cells were stimulated in both CD152^{-/-} and CD152^{WT} cultures, flow cytometric true-count analyses were performed with Fluoresbrite YG latex microspheres (Polysciences) 12 h after primary stimulation.

Measurement of cell cycling, cytokines, and apoptosis

Cell cycle progression was measured by labeling T cells with CFSE (Molecular Probes). In brief, 1 \times 10⁷ cells/ml were washed with PBS and stained with CFSE (5 μ M in PBS) for 6 min at room temperature in the dark. The reaction was stopped by resuspending the cells in RPMI 1640 medium with 10% FCS. TNF- α and IFN- γ were detected in culture supernatants by ELISAs using DuoSet (R&D Systems) with a lower detection limit of 20 pg/ml. Intracellular stainings were performed in fixed cells incubated with brefeldin A for 4 h before fixation unless specified otherwise. Fixation and permeabilization of cells was performed using the Cytofix/Cytoperm kit from BD Biosciences.

To reproducibly count cell numbers in stimulated WT and knockout (KO) cultures by FACS, Fluoresbrite YG latex microspheres 20.0 μ m (Polysciences) were used. A defined number of Fluoresbrite microspheres was added to cell culture suspensions previously stained for CD8-positive cells with a fluorescent-conjugated Ab. By comparing the known concentration of Fluoresbrite microspheres with the number of CD8-positive events, absolute cell counts could be evaluated. The binding of Annexin V-PE (BD Biosciences) was used to follow phosphatidylserine exposition on early apoptotic cells. The staining was performed according to the manufacturer's instructions. Briefly, 3 \times 10⁵ cells/ml were incubated with saturating concentrations of Annexin V-PE for 20 min at room temperature and immediately analyzed by flow cytometry. Late apoptotic cells were counter stained by propidium iodide (Invitrogen Life Technologies).

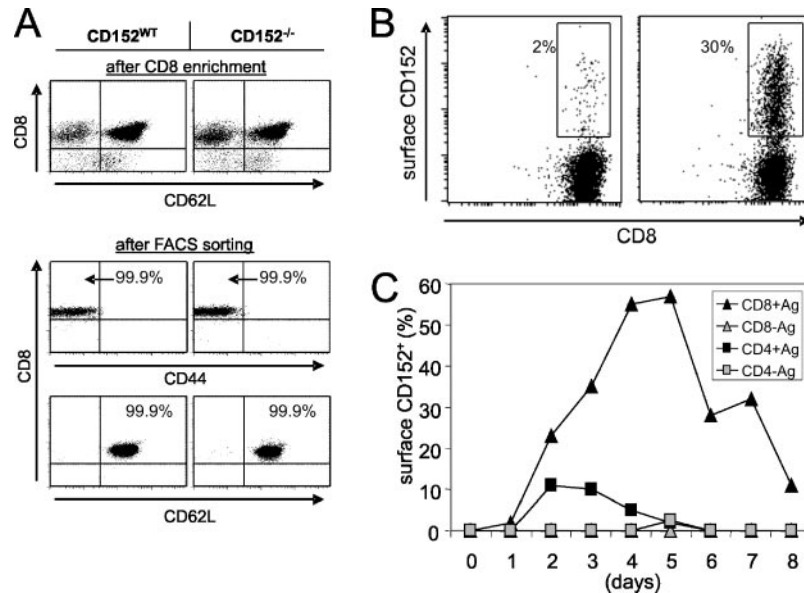


FIGURE 1. Ag-specific stimulated CD8 cells show higher frequencies of surface CD152⁺ cells compared with CD4 cells. *A*, Representation of naive CD8⁺CD44^{low} cell sorting of CD152-WT and -KO cells by FACS. The *upper panels* show the MACS-enriched CD8⁺ T cell population. The *lower panels* represent the subsequently naive sorted CD8⁺CD44^{low} fractions, which at the same time are CD62L⁺. *B*, Frequency of CD152 expression as measured by liposome immunostaining. Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from OT-1 mice were stimulated with the peptide OVA_{257–264} and congenic APCs, as described in *Materials and Methods*. Expression of surface CD152 was evaluated with liposome immunostaining on day 3 after primary stimulation, *left panel* shows control staining performed with isotype-matched Ab. *C*, Kinetics of CD152 surface expression on CD8 and CD4 cells as monitored by liposome immunostaining during days 1–8 after onset of primary stimulation. For analyzing CD152 expression on CD8 cells, naive cells were stimulated and analyzed as in *B*. Naive CD62L^{high}CD4⁺ T cells from OT-2 mice were isolated and stimulated with Ag and APC. Expression of surface CD152 was evaluated at time points after the onset of primary stimulation as indicated. Data in *C* show values subtracted from isotype controls. No CD152 was expressed in the absence of Ag. One of three independent experiments is shown.

Four-color cytometric analysis of surface expression of CD152

Surface expression of CD152 was detected using magnetofluorescent liposomes (17). T cells were incubated with unconjugated hamster anti-CD152 Ab at a concentration of 1 $\mu\text{g}/\text{ml}$ for 15–20 min at 4°C. Then, cells were washed twice and incubated with Cy5 dye-filled liposomes, conjugated to anti-ham Fab for 30 min at 4°C. Unbound liposomes were removed by washing the cells thrice with PBS-BSA. The specificity of CD152 staining was controlled by isotype control Ab conjugated with Cy5-filled liposomes as well as by incubation of cells with Cy5-filled liposomes only. Cytometric analyses were performed using a FACSCalibur (BD Biosciences) and FlowJo (Tree Star) software. Dead cells were excluded by forward and sideward scatter gating and propidium iodide staining in surface staining analyses.

Results

Large number of activated CD8 T lymphocytes express CD152 on the cell surface

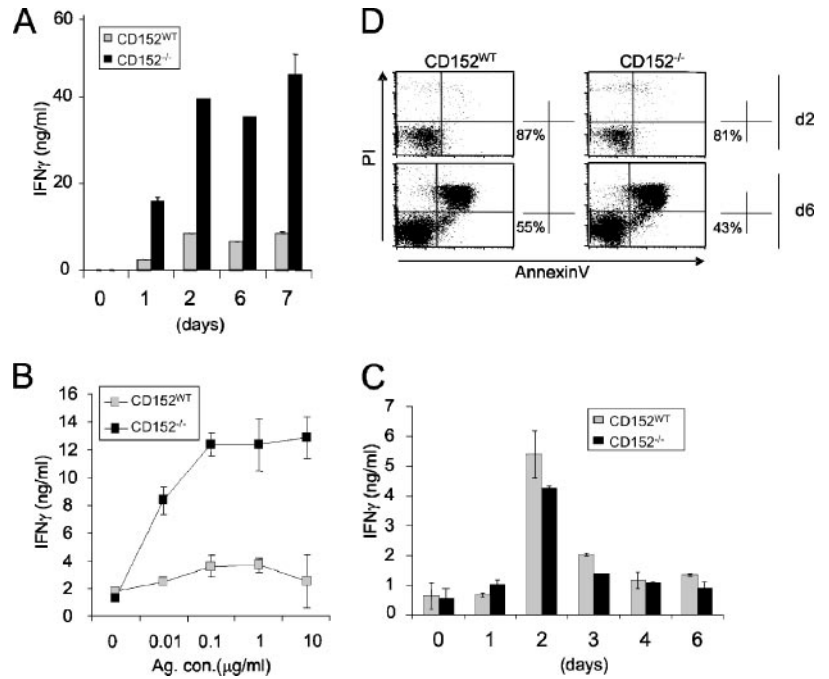
CD152 is not expressed on the surface of resting T cells and is difficult to detect even after stimulation of T cells (24, 28, 29). To analyze CD152 expression on the surface of CD4 and CD8 cells, we stimulated naive OVA-specific CD4⁺ or CD8⁺ TCR^{tg} T cells with their respective antigenic peptide OVA_{323–339} or OVA_{257–264}, respectively, in the presence of congenic APCs. Enrichment of primary, naive CD44^{low}CD8⁺ T cells resulted in a purity of $\geq 99.9\%$ (Fig. 1*A*), and enrichment of primary, naive CD62L⁺CD4⁺ T cells resulted in a purity of 99%. To unambiguously identify T cells expressing surface CD152, we used an enhanced staining technique (30) based on CD152-specific immunofluorescent liposomes, which increased the detection sensitivity of surface CD152 at least 1000-fold (31). Using this optimized method, expression of surface CD152 was evaluated at different time points after the onset of primary activation of CD4⁺ and CD8⁺ T cells (Fig. 1, *B* and *C*). Specificity controls revealed $< 2\%$ unspecific staining in Ag-stimulated T cells (Fig. 1, *B* and *C*). The surface

expression of CTLA-4 on CD8 cells starts 48 h after activation, reaches maximal expression with up to 60% CD152⁺ cells between day 4 and 5, and can still be detected at day 8 (Fig. 1, *B* and *C*). In contrast to this, the surface expression of CD152 on CD4 cells peaks already between day 2 and 3 when at most 12% CD152⁺ cells are detectable, rapidly declines, and is barely detectable on day 5 (Fig. 1*C*). Thus, both primary activated CD4 and CD8 T cells are able to express surface CD152, but it is expressed at a 2- to 6-fold higher frequency in CD8 cells and retained at the cell surface much longer, indicating that CD152 might play a more prominent role in CD8 cells.

CD152 down-regulates IFN- γ secretion of Ag-specific activated CD8 lymphocytes

IFN- γ is one of the most important effector cytokines of cytotoxic CD8 T cells, which has been shown to be unaffected by CD152 during primary stimulation (22). Naive CD8 cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated with Ag and congenic APCs, and concentrations of IFN- γ were monitored in the supernatants collected at different time points after the onset of a primary stimulation. To ensure that equal numbers of CD8 cells were stimulated in both CD152^{-/-} and CD152^{WT} cultures, flow cytometric analyses were performed with Fluoresbrite microspheres on CD8 TCR^{tg} T lymphocytes 12 h after primary stimulation. It was consistently observed at all time points analyzed (days 0–7) (Fig. 2*A*) that the IFN- γ concentration in supernatants of Ag-stimulated CD152^{-/-} CD8 lymphocytes were at least 5–7 times higher than those of CD152^{WT} CD8 lymphocytes. Cytometric detection of IFN- γ R revealed similar surface expression in both populations (data not shown). The titration of Ag for stimulation (0–10 $\mu\text{g}/\text{ml}$) showed that the IFN- γ concentration in supernatants was enhanced in CD152^{-/-} CD8 populations as compared with CD152^{WT} ones

FIGURE 2. CD8 T cell culture supernatants of Ag-specific stimulated CD152^{-/-} have higher concentrations of IFN- γ compared with CD152^{WT} cultures. Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated with the peptide OVA₂₅₇₋₂₆₄ (A, B, and D) or anti-CD3 (C) and congenic APCs. A, IFN- γ levels in the supernatant were evaluated after primary stimulation of CD8 T cells with the peptide OVA₂₅₇₋₂₆₄ at the time points indicated. B, Dose response of IFN- γ in the supernatant evaluated on day 4 after primary stimulation of CD8 T cells stimulated with indicated Ag concentrations. C, IFN- γ levels in the supernatant were evaluated after primary stimulation of CD8 T cells with anti-CD3 and congenic APCs at the time points indicated. D, Ag-induced cell death in CD152^{WT} and CD152^{-/-} CD8 T cells during primary stimulation. CD152^{WT} and CD152^{-/-} CD8 T cells undergo equal levels of activation-induced cell death during primary stimulation. Annexin V stainings were performed at the indicated time points. Data here represent one of two to four independent experiments.



in a dose-dependent manner (Fig. 2B). Because CD152 regulates IL-2 production in T lymphocytes and CD8 cells are dependent on IL-2 3 days after the onset of stimulation (23, 24), experiments were conducted both in the presence or absence of IL-2 addition for expansion of CD8 cells. IL-2 addition during expansion of CD8 cells did not affect CD152-mediated down-regulation of IFN- γ levels in CD152^{WT} cultures both after primary (Figs. 2A and 3A) and secondary stimulation (Fig. 3C), showing that the inhibitory effect of CD152 on IFN- γ is independent of IL-2. Enhanced cell death of Ag-specific stimulated CD152^{WT} CD8 cells was also excluded to account for lower IFN- γ concentrations in their culture supernatants (Fig. 2D). To further investigate whether the down-regulatory effect of CD152 on IFN- γ was dependent on a certain signaling quality of the TCR-complex, naive CD8 cells were stimulated with soluble anti-CD3 plus APCs, and supernatants were analyzed for IFN- γ at different time points after the onset of the primary stimulation. Results from ELISA showed that anti-CD3 plus APC-stimulated CD152^{WT} and CD152^{-/-} cultures have comparable levels of IFN- γ in their supernatants at all time points analyzed (Fig. 2C). Thus, CD152 regulates IFN- γ secretion both in primary and secondary stimulated CD8 cultures. However, this action depends on the quality of the TCR signaling cascade because only Ag-specific stimulated CD8 T cells showed this response.

CD152 down-regulates TNF- α levels in Ag-restimulated CD8 cultures

TNF- α is also an important effector and cytotoxic cytokine produced by CD8 cells, which has previously not been studied in relation to CD152. Because of its importance in apoptosis and cytotoxicity in CD8 cells (32), TNF- α was also monitored in the supernatants collected at different time points after primary stimulation of naive CD8 cells from CD152^{WT} or CD152^{-/-} OT-1 mice. ELISA results showed that at all time points analyzed (days 0–7), TNF- α levels in Ag-stimulated CD152^{-/-} CD8 cultures were comparable to those in CD152^{WT} cultures in the presence or absence of IL-2 (Figs. 3B and 4A). Corresponding results were likewise observed when CD152^{WT} or CD152^{-/-} CD8 T cells were stimulated with different concentrations of Ag (0–10 μ g/ml)

and TNF- α levels monitored on day 4 after primary stimulation (Fig. 4B). In contrast, when ELISAs were performed on supernatants collected after secondary Ag-specific stimulation, cultures of

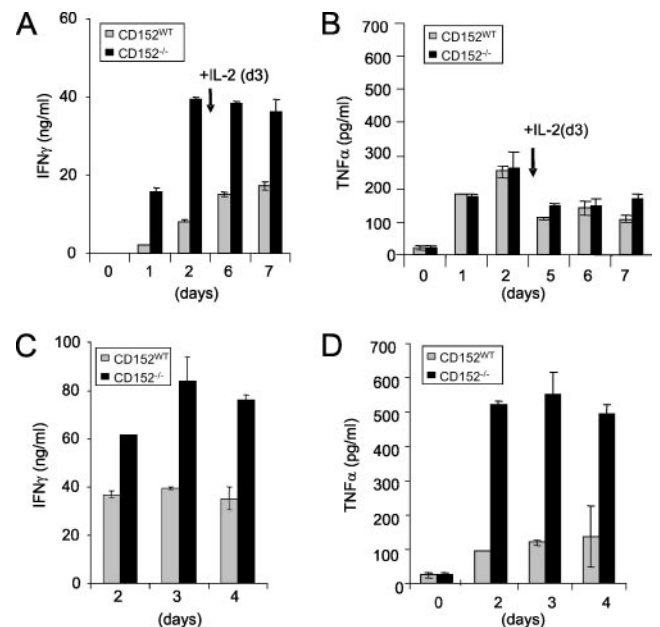


FIGURE 3. Ag-specific stimulated CD152^{-/-} CD8 culture supernatants show higher TNF- α only during secondary stimulation compared with CD152^{WT} cultures. The presence of IL-2 in culture supernatants is not influencing CD152-mediated effects on IFN- γ and TNF- α production. Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated by use of peptide OVA₂₅₇₋₂₆₄ and congenic APCs. IFN- γ and TNF- α concentrations in the supernatant were evaluated after primary stimulation with IL-2 added on day 3 (A and B) and after secondary stimulation with IL-2 added on day 0 (C and D) at the time points indicated. IFN- γ levels in the culture supernatants were analyzed by ELISA after primary (A) and after secondary stimulation (C). TNF- α concentrations were evaluated after primary (B) and after secondary stimulation (D). Data here represent results from one experiment of three independent experiments.

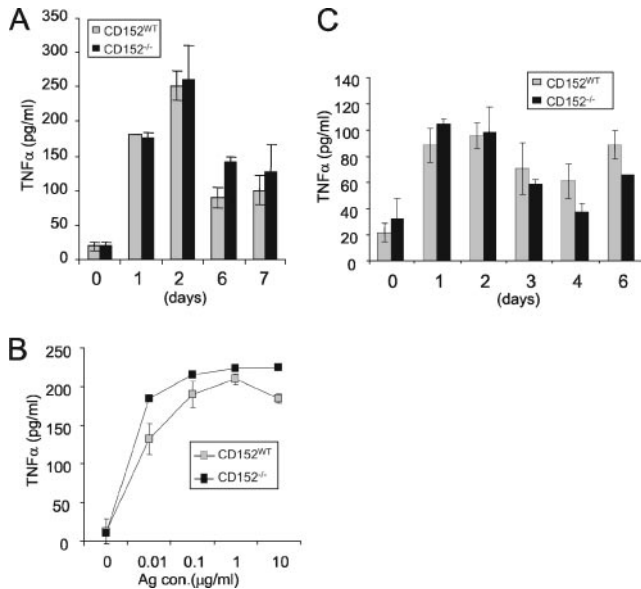


FIGURE 4. TNF- α concentrations are similar in CD152^{-/-} compared with CD152^{WT} CD8 culture supernatants after primary stimulation. Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated with the peptide OVA_{257–264} (A and B) or anti-CD3 (C) and congenic APCs. A, TNF- α levels in the supernatant were evaluated after primary stimulation of CD8 cells at the time points indicated. B, Dose response of TNF- α in the supernatant on day 4 after primary stimulation of CD8 cells stimulated with indicated Ag concentrations. C, TNF- α levels in the supernatant were evaluated after primary stimulation of CD8 T cells with anti-CD3 and congenic APCs at the time points indicated. Data represent results of more than three independent experiments.

CD152^{-/-} CD8 T cells reproducibly showed at all time points investigated dramatically higher amounts of TNF- α than those of CD152^{WT} cells (Fig. 3D). Naive CD8 cells were also stimulated with soluble anti-CD3 and APCs, and ELISAs were performed on

supernatants collected at different time points after primary stimulation. Results from ELISA also showed that CD3-stimulated CD152^{WT} and CD152^{-/-} cultures reproducibly have comparable levels of TNF- α in their supernatants at all time points (Fig. 4C). Thus, CD152 regulates TNF- α levels only in Ag-restimulated CD8 cells.

CD8 cell proliferation is not suppressed by CD152 during primary stimulation

With respect to proliferation, CD152 has been shown to regulate only secondary and not primary responses. This was shown by thymidin incorporation into all proliferating cells in a mixed APC-CD8 T cell culture (22). To investigate whether increased IFN- γ levels in primary CD152^{-/-} CD8 cultures were due to increased numbers of individual effector CD8 cells in the culture, proliferation was monitored in the CD152^{WT} and CD152^{-/-} cultures at different time points by CFSE labeling of naive cells during the onset of stimulation. CFSE is a vital fluorescent dye incorporated by cells during labeling. Proliferation reduces the intensity of the dye as CFSE is diluted to half in daughter cells. Thus, CFSE intensity can serve to measure the extent of cell divisions of individual cells. The results show that at all time points analyzed (day 0, 2, 4, and 6), there was substantial proliferation in both Ag (Fig. 5A) and CD3-stimulated (Fig. 5B) CD152^{-/-} and CD152^{WT} CD8 T cell cultures. Furthermore, proliferation capacities of both cultures were comparable in the presence or absence of IL-2 (Fig. 5C), which is in concert with previous observations (22). Experiments showed that CD152^{WT} or CD152^{-/-} CD8 cells, even when stimulated with different concentrations of Ag (0.01–10 μ g/ml), had comparable proliferation capacities as monitored by CFSE dilution on day 4 after primary stimulation (Fig. 5D). No spontaneous proliferation was observed in both populations without Ag (0 μ g/ml). These results show that proliferation was unaffected by CD152 in primary CD8 T cells, and that increased levels of IFN- γ in CD152^{-/-} cultures were not a result of increased numbers of effector cells.

FIGURE 5. Proliferation of Ag-specific stimulated CD152^{WT} and CD152^{-/-} CD8 cells is similar. Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated with the peptide OVA_{257–264} (A, C, and D) or anti-CD3 (B) and congenic APCs. A and B, Proliferation of CD8 T cells was evaluated by CFSE dilution at the time points indicated after primary stimulation with Ag (A) or anti-CD3 (B), respectively. C, Proliferation was evaluated at time points indicated by CFSE dilution after primary stimulation with IL-2 added during expansion on day 3 after primary stimulation. D, Dose response of CD8 cells were analyzed for proliferation using CFSE dilution analysis on day 4 after primary stimulation with indicated Ag concentrations. Data represent results of four independent experiments.

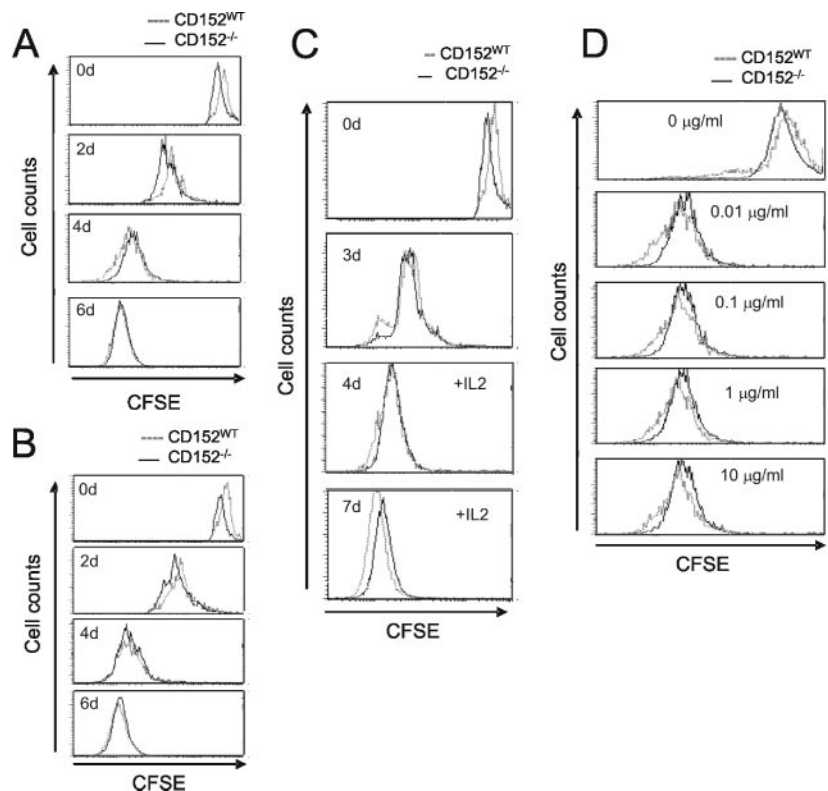
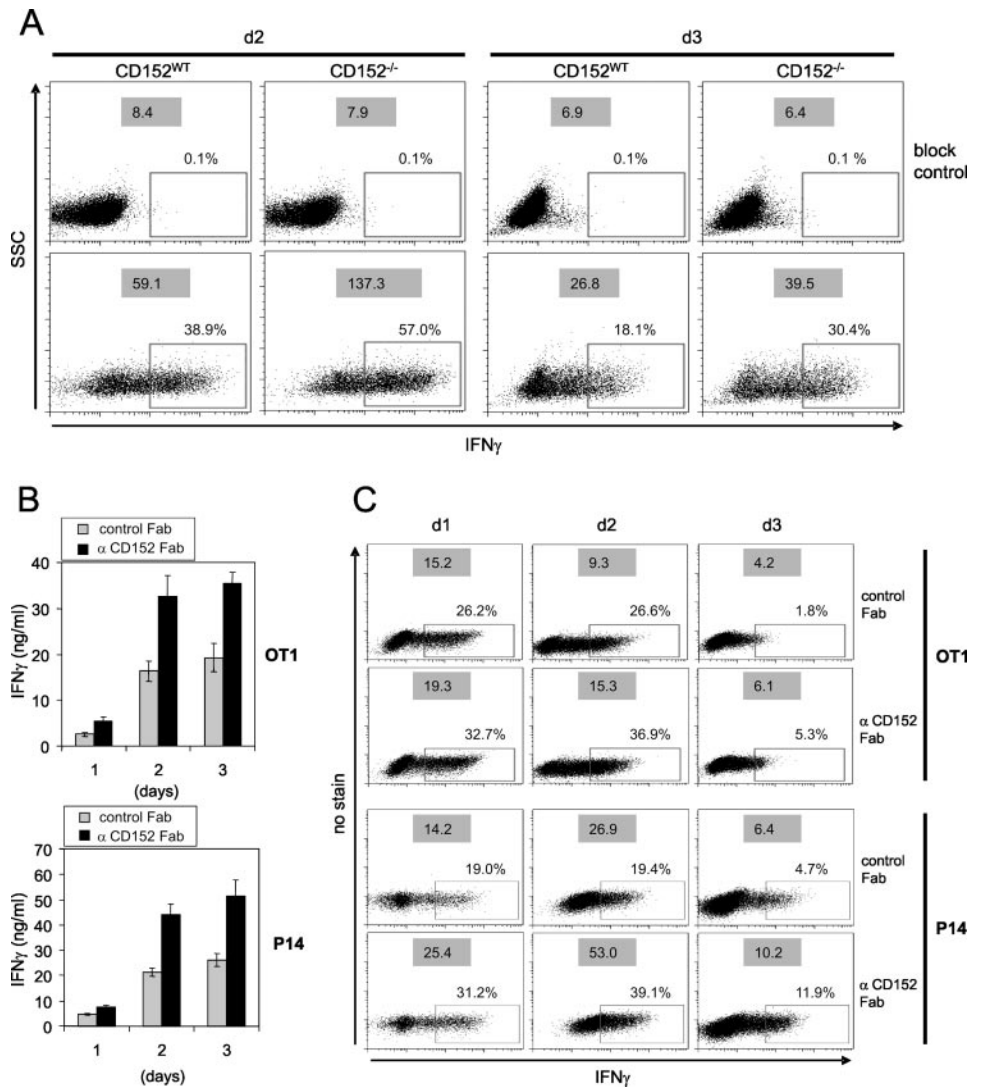


FIGURE 6. IFN- γ production by individual CD8 T lymphocytes is inhibited by CD152. **A**, Naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were stimulated by use of peptide OVA₂₅₇₋₂₆₄ and congenic APCs. Intracellular IFN- γ in CD152^{WT} (WT) and CD152^{-/-} (KO) was detected by flow cytometry on day 2 and day 3. Percentages refer to CD8⁺ T lymphocytes population. Before fixation, cultures were incubated with PMA, ionomycin, and brefeldin A for 4 h. Control stainings were performed by adding unconjugated Ab of the same specificity in 100-fold excess before staining with the conjugated Ab. **B**, Naive (CD8⁺CD44^{low}) CD8⁺ T cells from OT-1 mice (*upper panel*) and P14 mice (*lower panel*) were stimulated together with congenic APCs by use of peptide OVA₂₅₇₋₂₆₄ and GP33, respectively. Cultures were set up with either CD152-specific or control Fab added. Intracellular IFN- γ was detected by flow cytometry on day 1, 2, and 3. Percentages refer to CD8⁺ T lymphocyte population. Gray shaded numbers indicate IFN- γ MFI. **C**, Supernatants of the same cultures shown in **C** from OT-1 mice (*upper panel*) and P14 mice (*lower panel*) were analyzed for IFN- γ concentration by ELISA. One representative result of four independent experiments is shown.



Frequency of IFN- γ ^{high} producers is regulated by CD152 in CD8 cells during primary stimulation

Because augmented levels of IFN- γ in CD152^{-/-} cultures were not due to increased absolute CD8 effector numbers, it would have been possible that either increased frequencies of IFN- γ producers or increased secretion of IFN- γ per cell had caused this effect. To this end, intracellular amounts of IFN- γ were measured in primary CD152^{WT} and CD152^{-/-} CD8 populations at different time points (days 2 and 3) by intracellular cytokine stainings and subsequent flow cytometry (Fig. 6A). Intracellular IFN- γ was observed to be produced by a high fraction of CD8 T cells in both CD152^{-/-} and CD152^{WT} cultures. Interestingly, there was a consistent difference in the mean fluorescence intensity (MFI) of intracellular IFN- γ between CD152^{-/-} CD8 cells and those of CD152^{WT} (Fig. 6A, \square). Monitoring individual cells, the frequency of IFN- γ ^{high} producers was 40 to nearly 70% higher in CD152^{-/-} cultures than in CD152^{WT} ones on day 1, 2, 3, and 4 (Fig. 6A; data of day 1 and 4 are not shown). Thus, increased numbers of IFN- γ ^{high} producers might contribute to the observed enhanced IFN- γ production when CD152 is genetically inactivated.

To substantiate a role for CD152 in controlling IFN- γ production of CD8 cells, we confirmed our data using serological blockade of CD152 and also TCR^{tg} monoclonal T cells of another specificity (Fig. 6, B and C). Therefore, CD152 was inactivated on CD8

cells of OT-1 and P14 mice, respectively, by adding neutralizing anti-CD152 Fab to CD8 cells during primary stimulation, and IFN- γ production was monitored in the supernatants on day 1, 2, and 3 (Fig. 6, B and C). The specificity of the serological blockade was controlled by adding control Fab to the cultures (Fig. 6B, \square ; Fig. 6C, *upper panels*). Following Ag-specific stimulation of CD8 cells of OT-1 and P14 mice, CD8 cells of both TCR^{tg} mice that were activated under serological blockade of CD152 produced >2-fold more IFN- γ than CD8 cells that received a CD152 signal (Fig. 6B). Detection of IFN- γ producers was performed by analyzing intracellular IFN- γ of CD8 cells without prior PMA/ionomycin stimulation. Monitoring individual cells, the frequency of IFN- γ ^{high} producers is 6–20% higher in CD152^{-/-} cultures than CD152^{WT} ones on day 1, 2, and 3 (Fig. 6C). Thus, higher numbers of IFN- γ ^{high} producers might contribute to the observed enhanced IFN- γ production when CD152 is serologically inactivated.

To determine that the observed CD152-mediated inhibition of IFN- γ production of CD8 T cells during primary stimulation is not an indirect effect, e.g., mediated by non-CD8 cells participating in the culture, we performed experiments in which CD152^{WT} and CD152^{-/-} CD8 T cells were cocultured. For this test, we labeled either naive CD8 cells from CD152^{WT} or CD152^{-/-} OT-1 mice with CFSE and cocultured them together with their respective unlabeled counterpart CD8 cells. Cultures were stimulated with Ag

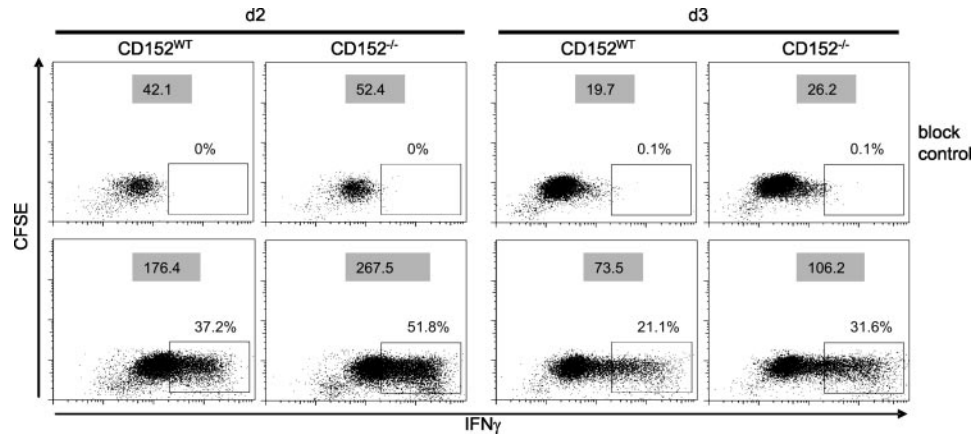


FIGURE 7. CD152-mediated inhibition of IFN- γ production is independent of culture conditions. Coculture: naive (CD8⁺CD44^{low}) OVA-specific CD8⁺ T cells from CD152^{WT} and CD152^{-/-} OT-1 mice were jointly stimulated by use of peptide OVA₂₅₇₋₂₆₄ and congenic APCs. Either CD8⁺ T cells from CD152^{WT} or CD152^{-/-} OT-1 mice were labeled with CFSE. To exclude effects of CFSE labeling on cell metabolism, only the CFSE-negative population was analyzed (CFSE-positive events are not shown). Instead, this experiment was performed vice versa with either CD152^{WT} or CD152^{-/-} OT-1 cells labeled with CFSE to get comparable results. Intracellular IFN- γ in CD152^{WT} (WT) and CD152^{-/-} (KO) was detected by flow cytometry on day 2 and 3. Percentages refer to the CFSE-unlabeled CD8⁺ T lymphocytes population. Before fixation, cultures were incubated with PMA, ionomycin, and brefeldin A for 4 h. Gray shaded numbers indicate IFN- γ MFI. Control stainings were performed by adding unconjugated Ab of the same specificity in 100-fold excess before staining with the conjugated Ab. One of two independent experiments is shown.

and congenic APCs. To exclude inhibitory effects of CFSE labeling on production of IFN- γ , the same experiment was conducted vice versa each time analyzing the CFSE⁻-unlabeled CD8 T cells. By using this experimental approach, we were able to compare intracellular IFN- γ levels of CD152^{WT} and CD152^{-/-} CD8 T cells in the same culture by distinguishing the two populations according to CFSE staining. On days 1 and 2 after primary stimulation, intracellular IFN- γ in CD8 T cells was detected by flow cytometry. As shown in Fig. 7, coculturing of CD152^{WT} and CD152^{-/-} CD8 T cells did not influence the CD152-mediated inhibitory effect on IFN- γ ^{high} producers.

Discussion

We show in this study for the first time that CD152 down-regulates the major effector cytokine IFN- γ in primary and secondary stimulation of CD8 cells, whereas TNF- α is only regulated by CD152 upon restimulation of CD8 cells. The inhibition mediated by CD152 on IFN- γ production of CD8 cells was shown to be independent of IL-2 or other soluble factors and is therefore cell-autonomous. Also, enhanced numbers of effector cells in CD152^{-/-} CD8 cells were not the cause of enhanced cytokine production because proliferation was similar in WT and T cells with genetically inactivated CD152. Monitoring individual cells expressing IFN- γ upon stimulation revealed that more IFN- γ ^{high} cells were present when CD152 was inactivated. These novel functions in CD8 cells also have implications on CD152's role in the generation of memory CD8 cells. Interestingly, we found that surface CD152 was expressed in much higher frequencies in individual, activated CD8 than Ag-stimulated CD4 cells. Our study showing the frequencies of CD152-expressing cells using the sensitive liposome technique, detecting as few as 100 molecules per cell, demonstrated that populations of Ag-specific stimulated CD8 cells have five times more CD152⁺ cells than CD4 cells and that the CD152 molecule was retained much longer on their surface than on CD4 cells. Hence, apart from activation and differentiation histories of an activated naive T cell, CD152 surface expression is also a cell intrinsic effect in which level and duration of expression depends on the T cell lineage. The higher fraction of CD152-expressing cells in CD8⁺ than in CD4 populations in our study was

probably not mediated by CD8-derived cytokines because it was reported previously that most cytokines, except IL-2 to a certain extent, did not markedly up-regulate CD152 (33). We found that even without addition of IL-2 to CD8 cultures during expansion, there was still a higher expression of surface CD152 on CD8 cells than on CD4 cells (data not shown). Moreover, cocultures of cells of both lineages propagated in the presence of IL-2 still resulted in a higher frequency of CD152⁺CD8 than CD4 T cells (data not shown). This shows that the expression of surface CD152 on CD8 T cells is a cell-intrinsic effect.

The functional relevance of this higher expression of CD152 and also its longer retention of the molecule on the surface of CD8 than on CD4 cells is largely unknown. But because CD152 has different functions in CD8 and in CD4 cells (e.g., in anergy induction), which at present are not precisely elucidated, this differential expression may be relevant in functions not performed by CD4 cells. Longer retention of CD152 on CD8 cells can mean that CD152 has an important function in much later phases of this cell type.

It has previously been shown that CD8 T cells from WT and 2C TCR transgenic mice express CD152 on the cell surface upon activation in vitro and that the proliferative response to anti-CD3 and anti-CD28 in vitro can be inhibited by additional CD152 cross-linking (14, 23). Although CD152 regulates CD8 cells, as far as antigenic responses are concerned, it has been implicated only in secondary CD8 responses (22). However, our studies for the first time illustrate that CD152 can also mediate its regulatory role during primary stimulation of CD8 cells with respect to IFN- γ production. We have shown that genetic inactivation of CD152 in Ag-specific CD8 cells up-regulates IFN- γ levels in the cultures dramatically. This effect of CD152 is probably not due to the overall activated status of ex vivo CD152^{-/-} CD8 cells because only naive cells were isolated on the basis of their CD44^{low} expression both in CD152^{WT} and CD152^{-/-} T cells, with the same stringent electronic gating during FACS sorting. Moreover, in these CD152^{-/-} OT-1 mice, most CD8 cells are naive and are not activated due to CD152 deficiency until the age of 7 wk (14, 22). Thus, it is unlikely that CD8 cells from CD152-deficient mice have

higher levels of the effector cytokine IFN- γ due to enhanced activation status during the onset of stimulation compared with CD152^{WT} cells. Our experiments showing that CD152^{WT} cells cocultured with CD152^{-/-} CD8 cells still have lower numbers of IFN- γ producers, rule out the possibility that a soluble factor, possibly secreted by hyperactivated CD152^{-/-} cells, is responsible for the overproduction of IFN- γ . Moreover, TNF- α is shown to be unaffected by CD152 in primary CD8 cells, confirming similar activation status of CD152^{-/-} and CD152^{WT} CD8 T cells, respectively.

Detection of IFN- γ production by individual cells of activated, Ag-specific primary CD8 cells from CD152^{-/-} and CD152^{WT} mice reveal that almost all the cells from both the cultures produce the cytokine, but, interestingly, there is a population of IFN- γ ^{high} producers whose frequency is lower in CD152^{WT} CD8 cultures than in CD152^{-/-} cultures. This decrease in the frequency of effectors under the influence CD152 implies that CD152 regulates a small population of cells that may have an important functional relevance. This may reflect heterogeneity of T cells with respect to CD152 functions (17, 34), which may be a result of differential signaling of CD152 within a T cell population, depending possibly on its high or low expression levels on the surface of CD8 T cells.

Our data show that IFN- γ ^{high} producers are regulated by CD152 signaling. Our finding is consistent with the concept that T cells receiving the strongest TCR signal express CD152 at the cell surface, which suggests that these cells are inhibited by CD152, and thus prevent the dominate response and avoid epitope spreading (28). It might also be that the cells expressing surface CD152 are the fittest (35) and are tightly controlled by CD152 signals to become memory cells (36, 37). Recently, it was reported that, in terms of Th1 memory responses, there are distinct lineages of Th1 cells with different capacities to develop into memory cells. Accordingly, IFN- γ ^{neg/low} cells survive and develop into long-lived memory cells providing the overwhelming majority of long-term resting memory cells, in contrast to cells producing high amounts of IFN- γ during primary responses. Considering our recent data that CD152 signaling protects CD4 Th cells from apoptosis (18), this is another way in which CD152 signaling could contribute even more to memory formation. Thus, heterogeneity of CD8 cells with respect to CD152 expression and signaling, associated with heterogeneity of IFN- γ production, possibly contributes to long-term memory cell generation.

It is also interesting to note that only IFN- γ but not TNF- α is down-regulated by CD152 in primary stimulated CD8 cells, illustrating independent regulation of TNF- α and IFN- γ production by CD152 and hence bifurcation of CD152 signals. However, the fact that both of the cytokines are down-regulated dramatically by CD152 during secondary stimulation, rules out the argument that CD152 does not influence TNF- α at all. However, TNF- α not being regulated by CD152 in primary but only in secondary stimulated CD8 cells, raises the possibility that CD152 acts on TNF- α signaling depending on the activation history of CD8 cells and their CD152 surface expression. Another possible explanation is that very strong negative signals are needed to regulate TNF- α compared with those needed to down-regulate IFN- γ . Thus, it is possible that, in a given T cell population, only high CD152 expression or high frequency of CD152⁺ cells can generate a strong enough negative CD152-mediated signal that could regulate TNF- α production. Because high frequencies of CD152⁺ cells are achieved earlier (day 2) in secondary than in primary stimulated CD8 cells and TNF- α is also only up-regulated at an early phase of an immune response, this could explain why TNF- α is down-regulated only in secondary stimulation. This result already illustrates the differential regulation of two different cytokines by

CD152 signals. Another interesting observation from proliferation assays is that both CD152^{-/-} and CD152^{WT} cells proliferate with an equal number of cell divisions in all Ag- and CD3-stimulated CD8 T cell cultures as assessed by CFSE dilution assay. This result is a further indication that regulatory signals emanating from CD152 also bifurcate to control proliferative pathways and cytokine signals differentially.

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Disclosures

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